ORIGINAL ARTICLE

Clinico-pathological correlation of micronuclei in oral squamous cell carcinoma by exfoliative cytology

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ABSTRACT
Oral squamous cell carcinoma accounts for 90% to 95% of all oral malignancies. Though its diagnosis seldom presents difficulty, it is the cancer staging and histopathological grading that are important to prognostication; and micronuclei are good prognostic indicators. Micronucleus frequencies in oral exfoliated cells stained with papanicolaou stain were counted and correlated with the histopathological grades and clinical stages of squamous cell carcinoma patients. They were also compared with healthy control subjects. Micronuclei (MN) frequencies were found higher in squamous cell carcinoma patients than in control subjects. MN frequencies were also found to be raised with increasing histological grades of squamous cell carcinoma.
Key words: Exfoliative cytology, micronuclei, oral squamous cell carcinoma

INTRODUCTION

Squamous cell carcinoma is by far the most common oral mucosal malignant tumor. Though the diagnosis of oral squamous cell carcinoma seldom presents difficulty, it is the cancer staging and histopathological grading that are more important for prognostication; and micronuclei are good prognostic indicators. Different diagnostic methods such as routine histopathology (H and E-stained sections), exfoliative cytology, and immunohistochemistry are available today. Out of these, oral exfoliative cytology is particularly valuable for mass screening purposes. It has been used in the detection of oral squamous cell carcinoma and has been shown to have a sensitivity of 94%, specificity of 100%, and an accuracy of 95%.[1] It is the nucleus that expresses the genotypic alterations caused in the process of malignancy; and exfoliative cytology is a method that gives better insight of the nuclear changes in individual cells.

Micronuclei are extranuclear cytoplasmic bodies. They are induced in cells by numerous genotoxic agents that damage the chromosomes. The damaged chromosomes, in the form of acentric chromatids or chromosome fragments, lag behind in anaphase when centric elements move towards the spindle poles. After telophase, the undamaged chromosomes, as well as the centric fragments, give rise to regular daughter nuclei. The lagging elements are included in the daughter cells, too, but a considerable proportion is transformed into one or several secondary nuclei, which are, as a rule, much smaller than the principal nucleus and are therefore called micronuclei.[2] Bigger micronuclei result from exclusion of whole chromosome following damage to the spindle apparatus of the cell (aneugenic effect), whereas smaller micronuclei result from structural aberrations; causing chromosomal fragments (clastogenic effect).

Micronuclei are induced in oral exfoliated cells by a variety of substances, including genotoxic agents and carcinogenic compound in tobacco, betel nut, and alcohol.[3] Tobacco-specific nitrosamines have been reported to be potent clastogenic and mutagenic agents which are thought to be responsible for the induction of chromatid/chromosomal aberrations resulting in production of micronuclei.[3] The genotoxic and carcinogenic chemicals released from betel nut and tobacco and also the calcium hydroxide content of lime present in the betel quid are thought to be responsible for promotion of reactive oxygen species from areca nut extracts. These reactive oxygen species can in turn cause damage to the DNA.[3]

With this view in mind, the present study was carried out to assess the levels of micronuclei in oral exfoliative cytology of healthy control subjects and oral squamous cell carcinoma patients.

Aims and objectives

1. The present study was aimed at evaluation of frequency of micronuclei in papanicolaou stained smears of oral exfoliated cells from healthy control subjects and oral squamous cell carcinoma patients.
2. Comparison of micronucleus frequencies between the control group and the patients with different grades of oral squamous cell carcinoma.
3. To correlate, if any, between the micronucleus frequency in

oral exfoliated cells and the clinical stage and histological grade of squamous cell carcinoma.

MATERIALS AND METHODS

Patient selection

Subjects with oral lesions suspected to be malignant were selected as the study group. Relevant history of each patient, including their oral habits, was recorded thoroughly. Only those patients who were subsequently diagnosed histopathologically as having squamous cell carcinoma (SCC) and who had not received any therapy prior to study were included in the SCC group. Age- and sex-matched healthy subjects having no obvious oral lesions or habits of consumption of tobacco, other tobacco-related substances, or other such substances were selected as control group. Written informed consents from these patients were taken for the procedures to be carried out on them subsequently.

The subjects for the study were grouped as follows:
Group I (SCC): Thirty patients having oral squamous cell carcinoma, with an age range of 24 to 75 years.
Group II (Control): Twenty healthy control subjects, age and sex matched.

Collection of exfoliated cells

Subjects were asked to rinse their mouth gently with water. Oral mucosal cells were scraped from lesional tissue of squamous cell carcinoma group and from buccal mucosa of control group using a slightly moistened wooden spatula. The cells were immediately smeared on precleaned microscopic slides. Just prior to drying, the smears were fixed with commercially available spray fixative (available with the RAPIDPAP™ kit) for 15 min. The slides were coded and preserved in dust-free boxes until evaluation.

Biopsy procedure

Incisional biopsies were taken from the representative sites taking all aseptic precautions. The tissue specimens were labeled, fixed in 10% formalin for 24 hours, and paraffin embedded. The wax blocks were cut to obtain two tissue sections of 4-µm thickness for each block. The sections were stained by hematoxylin and eosin (H and E).

Histopathological grading

All the slides were observed under light microscope so as to see changes in epithelium, basement membrane, and connective tissue. Histopathological grading of squamous cell carcinoma was done according to the malignancy grading system proposed by Anneroth *et al.*[4] The slides were examined for various parameters such as degree of keratinization, nuclear polymorphism, number of mitoses per high-power field, break in the basement membrane, pattern of invasion, depth of invasion, inflammatory infiltrate, etc. Each parameter was scored according to its extent as points 1, 2, 3, or 4. Total malignancy point score for each patient was calculated by adding the points for each parameter and dividing the sum by the number of parameters examined. The final grading was done as follows.

Total malignancy point score between 1.0 and 2.0 = Grade I SCC; between 2.1 and 3.0 = Grade II SCC; and between 3.1 and 4.0 = Grade III SCC.

Clinical staging

The clinical staging of patients suffering from oral squamous cell carcinoma was done based on the site, size and extent of the lesion, and involvement of lymph nodes as per the TNM classification given by the American Joint Committee for Cancer Staging and End Results Reporting (AJCCS).[5]

Cytological staining and evaluation

All the cytological smears were stained by papanicolaou technique using a commercially available staining kit RAPIDPAP™ (Biolab Diagnostics, Tarapur, Maharashtra). The slides were mounted with cover glass using DPX mountant. All the slides were observed under light microscope using low magnification (×100) for screening and high magnification (×400) for counting of micronuclei.

Scoring criteria

The most commonly used method, i.e., the zigzag method, was followed for screening of slides. One thousand cells with intact nuclei and cell boundaries were counted on each slide. The criteria for designating an extranuclear body as 'micronucleus' were as follows:

1. Diameter less than one third of the main nucleus.
2. Staining intensity similar to, or slightly weaker than, that of the nucleus.
3. Round-to-oval shape.
4. Texture same as that of the main nucleus.
5. Close proximity but no actual contact with the nucleus.
6. Plane of focus same as that of the main nucleus.

Only those structures fulfilling the above-mentioned criteria were recorded as micronuclei. Micronucleated cells were counted out of 1000 intact epithelial cells, and they were expressed as percentages.[3,6]

The distribution of cases was done after applying the histological criteria and the TNM staging of each case, and the frequency of micronuclei in each case was recorded for further analysis. The statistical evaluation of the data obtained was done using unpaired *t* test.

RESULTS

The present study comprised of 30 cases of histologically diagnosed squamous cell carcinoma and 20 healthy control subjects without any habits of consumption of tobacco, other tobacco-related substances, or other such substances.

The age- and sex-wise distribution of squamous cell carcinoma cases was as follows. There were 10 (33.33%) cases of squamous cell carcinoma in the fifth decade, 9 (29.97%) cases in the seventh decade, and 5 (16.65%) cases in the sixth decade. Sixty percent of patients were males, while 40% were females.

The results obtained were analyzed for clinical staging, histopathological grading, and cytological evaluation as follows:

Clinical staging

The present study comprised of 18 (60%) cases of stage III and 12 (40%) cases of stage IV squamous cell carcinoma. There were no cases in stage I and stage II.

Histological grading

Out of 30 cases examined, 11 (36.66%) cases were of grade I carcinoma, 18 (60%) cases were of grade II carcinoma, whereas only 1 (3.33%) case was of grade III carcinoma.

Results of cytological evaluation

Stained cytological smears were screened for 1000 intact epithelial cells per slide for micronuclei as per the criteria mentioned earlier. [Figures 1-3] In the present study, frequencies of micronuclei were estimated in the control group and squamous cell carcinoma group.

Frequency of micronuclei ranged from 0% to 0.5% in the control group, whereas it varied from 1.1% to 3% in the SCC group. The mean micronucleus frequency was found to be significantly high among SCC group compared to controls ($P < .001$) [Table 1 and Graph 1].

In the present study, the values of micronucleus frequency within the SCC group were correlated with the histological grading and clinical staging. For this purpose, the micronucleus frequencies were compared within different clinical stages and histological grades. The frequencies of micronuclei in different histological grades of SCC are tabulated in Table 2. The micronucleus frequencies were found to increase from grade I to grade II, the difference being statistically highly significant ($P < .001$). The micronucleus frequency in grade III was observed to be higher than that in grade II. Since there was only 1 case in grade III, the statistical test could not be applied for comparison between grade II and grade III [Table 2 and Graph 2].

In the SCC group, the frequencies of micronuclei in different clinical stages were observed to be as follows. There were no cases in stage I and stage II. In stage III, mean micronucleus frequency was observed to be 1.778% ± 0.471%, whereas that in stage IV was 1.942% ± 0.464%. The micronucleus frequency was observed to increase from stage III to stage IV, but the difference was not statistically significant ($P = .3557$). Since there were no cases in stage I and stage II, statistical test could not be applied for the comparison. Thus it was observed that the frequency of micronuclei did not correlate with the clinical stages of SCC [Table 3 and Graph 3].

DISCUSSION

Squamous cell carcinoma of the oral mucosa accounts for 90% to 95% of all oral malignancies.[3] Oral exfoliative

Table 1: Comparison of micronuclei frequencies between squamous cell carcinoma group and control group

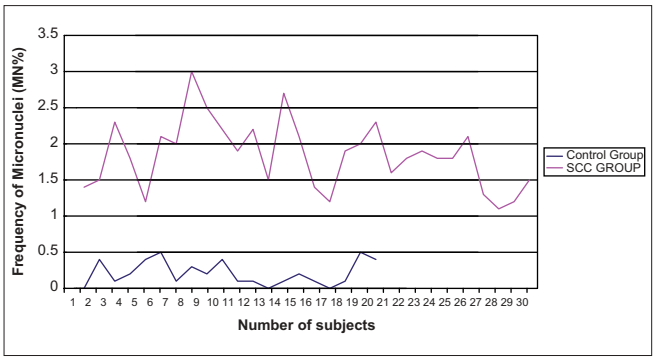
Study group	No. of cases	Males (%)	Females (%)	MN range %	Mean MN% ± SD	't' value	'P' value	Statistical significance
SCC group	30	18 (60)	12 (40)	1.1-3.0	1.843 ± 0.467	14.95	<0.001	-
Control group	20	11 (55)	9 (45)	0.0-0.5	0.210 ± 0.168	-	-	Highly significant

Table 2: Comparison of micronuclei frequencies amongst histological grades of squamous cell carcinoma

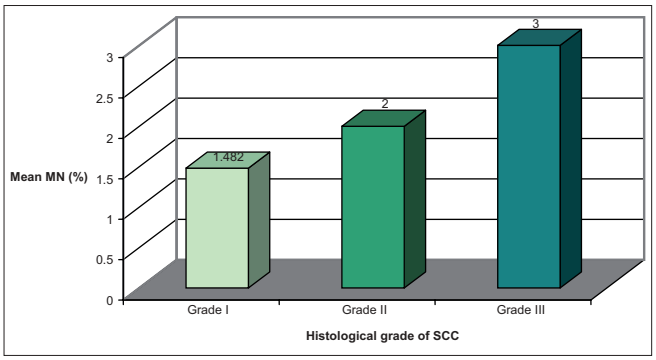
Histological grade	No. of cases	Mean MN% ± SD	't' value	'P' value	Statistical significance
Grade I	11	1.482 ± 0.286	-	-	-
Grade II	18	2.000 ± 0.368	3.9854	<0.001	Highly significant
Grade III	1	3.000	-	-	-

Table 3: Comparison of micronuclei frequencies amongst clinical stages of squamous cell carcinoma

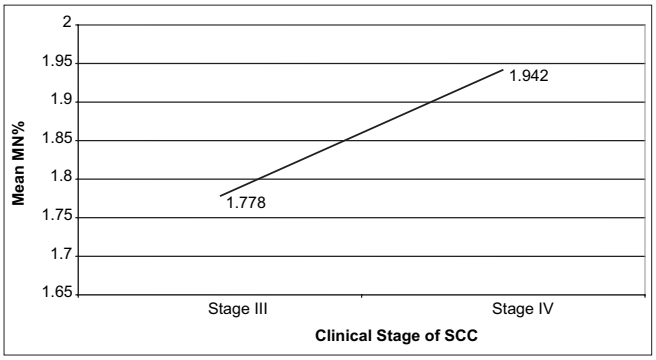
Clinical stage	No. of cases	Mean MN% ± SD	't' value	'P' value	Statistical significance
Stage I	-	-	-	-	-
Stage II	-	-	-	-	-
Stage III	18	1.778 ± 0.471	0.9391	0.3557	Not significant
Stage IV	12	1.942 ± 0.464	-	-	-



Graph 1: Comparison of micronucleus frequencies between control subjects and squamous carcinoma patients



Graph 2: Comparison of micronucleus frequencies between different histological grades of squamous cell carcinoma



Graph 3: Comparison of micronucleus frequencies in different clinical stages of squamous carcinoma

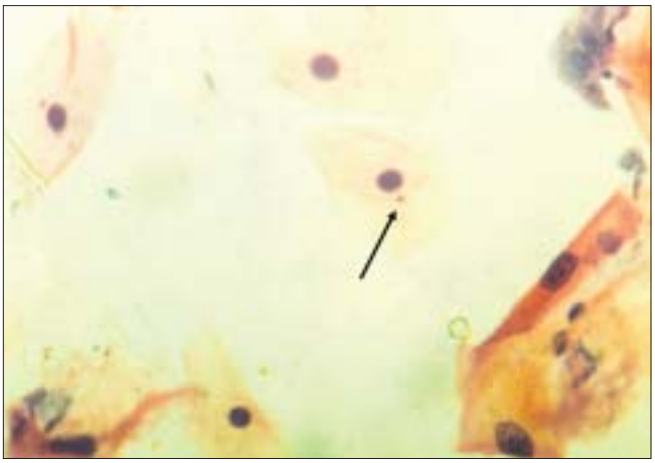


Figure 1: Exfoliated cells with single micronucleus (PAP stain, ×400)

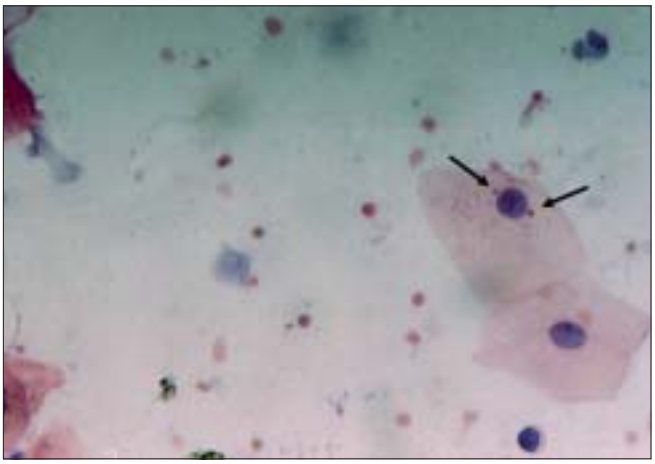


Figure 2: Exfoliated cells with two micronuclei (PAP stain, ×400)

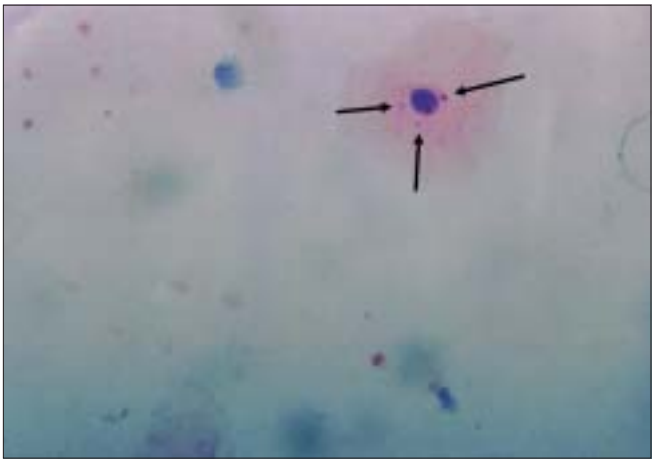


Figure 3: Exfoliated cells with three micronuclei (PAP stain, ×400)

cytology has been used extensively for screening cellular alteration in oral squamous cell carcinoma cases. An accuracy of 95% and a reliability of more than 96% in detection of squamous cell carcinoma in mass screening have been reported in the literature.[1]

Oral exfoliative cytology can reveal various cellular alterations in squamous cell carcinoma. It includes karyorrhexis, karyolysis, micronucleus formation, pyknosis, binucleation, broken-egg nucleus, anucleation, etc.[7,8] Micronuclei in oral exfoliated cells is a marker of chromosomal damage caused by genotoxic agents from tobacco and tobacco-related substances, alcohol, etc.[9] The micronucleus assay has been used to assess the genotoxic damage in oral squamous cell carcinoma and oral premalignancies.[10,11] The MN assay has been reported to correlate well with the histological grading of oral squamous

cell carcinoma and leukoplakia.[5] Incidence of micronuclei has been analyzed by various studies in normal patients, oral premalignancies, and oral squamous cell carcinomas.[3,12-14]

In the present study, maximum number of cases were observed to involve the buccal vestibule (15 cases), which was actually the site of placement of betel quid in most of the patients.

In the present study, micronuclei in oral exfoliated cells in the control group were observed to be in the range of 0% to 0.5% with a mean micronucleus frequency of 0.210% ± 0.168%. Various studies have evaluated the frequency of micronuclei in healthy control subjects from different population groups. The micronucleus frequency levels in control subjects in the present study were quite similar to those reported by earlier studies, such as 0.186% ± 0.026%,[15] 0.190% ± 0.01%,[16] 0.18%,[17] and 0.2%.[18]

The overall level of micronuclei in the SCC group was observed to be in the range of 1.1% to 3.0% in the present study, whereas it ranged from 1.4% to 9.15% in the SCC group as obtained by Kumar V *et al.*[3] Thus the levels in the present study were slightly lower than those reported by Kumar V *et al.* In the present study, exfoliated cells were utilized to make the smears for micronucleus analysis. Exfoliative cytology does not include the cells from the basal layer, which actually has the cells in the dividing stage that are more prone to genotoxic damage. These cells that mature and reach to the most superficial layer can then become available for screening when exfoliative cytology is executed. Therefore, the smears made by exfoliative cytology may have less chance of including micronucleated cells. This may be the reason for slightly lower levels of mean micronucleus frequency in the present study. In the study by Kumar V *et al.*, the cells from SCC lesions were obtained by mincing the biopsy tissue and preparing single cell suspensions from it. Since after mincing and making cell suspensions the smears include cells from all the epithelial layers (basal layer to superficial), the smears have more chance of including micronucleated cells. The other reason for higher levels in the study by Kumar V *et al.* may be that they had followed a fluorescent-acridine orange staining method and the analysis was done under fluorescence microscope, increasing the specificity to identify DNA-containing structures. This technique is a time consuming method, and it requires costlier chemicals and equipment. Therefore, in the present study, rapid papanicolaou technique was used in place of fluorescent dyes for staining purpose since it is very simple to use, less time consuming, and economical.

Another observation in the study by Kumar V *et al.* was that the frequency of micronuclei increased significantly from grade I to grade II to grade III respectively in squamous cell carcinoma group. Thus the MN frequency correlated well with the histological grading of squamous cell carcinoma. Similar results were obtained for increasing grades of SCC in the present study.

A significant ($P < 0.05$) stepwise increase was found in the percentage of micronucleated cells and micronuclei from control to pre-cancer patients, and from pre-cancer to cancer patients in a study by Saran *et al.*[19]

patients were grouped according to clinical stages of SCC in the present study. The frequencies of micronuclei were observed to be higher in stage IV as compared to stage III. Though the frequency of micronuclei was observed to be increasing from stage III to stage IV, the difference was not statistically significant. There were no cases in clinical stages I and II, and therefore a comparison could not be done between stages I, II, III, and IV. Thus the MN frequency in oral exfoliated cells did not correlate well with the clinical staging of squamous cell carcinoma as observed in the present study. However de Carvalho *et al.*[20] studied the frequency of micronucleus of the oral mucosa in 27 untreated patients with carcinoma of the oral cavity and oropharynx and found a higher micronucleus frequency in the stages T3 and T4 ($P = 0.01$) as compared to stages T1 and T2.[21]

A significant correlation of MN frequency with histopathological grading was observed in this study. However, the following limitation of this study was noticed. Routine histopathological grading is subjective and it depends upon the individual experience and assessment of microscopic observations. Since the histopathological grading of SCC is correlated with the MN frequency in the present study, the reliability of the results decreases if the grading itself is not accurate. Therefore, definite histopathological criteria should be followed to eliminate or minimize subjectivity and inter-individual variability. In the present study, the criteria laid down by Anneroth *et al.*[4] were followed for histopathological grading. It is needed that these criteria be tested on large samples and standardized for achieving unequivocal histopathologic grading of squamous cell carcinoma.

CONCLUSION

1. The mean micronucleus frequency in oral exfoliated cells was significantly increased in oral squamous cell carcinoma (SCC) group as compared to the control group.
2. Amongst the different histological grades, the mean MN frequency statistically significantly increased from grade I to grade II to grade III.
3. The mean micronucleus frequency did not increase significantly from clinical stage III to stage IV.
4. There was a statistically significant correlation of mean MN frequency with the histological grades of squamous cell carcinoma.
5. There was no statistically significant correlation of mean MN frequency with the clinical stages of squamous cell carcinoma.

Thus from the present study, it is evident that the percentage of micronuclei is uniformly elevated in all histologic grades of

oral squamous cell carcinoma, suggesting a strong cytogenetic damage of the oral epithelium. This cytogenetic damage may be because of the genotoxic and carcinogenic agents released from areca or tobacco products. Hence the micronucleus assay can be used as a biomarker of genotoxicity in predicting the effects of cancer intervention.

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